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Activation of Six1 target genes is required for sensory placode formation

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ABSTRACT

In vertebrates, cranial placodes form crucial parts of the sensory nervous system in the head. All cranial placodes arise from a common territory, the preplacodal region, and are identified by the expression of Six1/4 and Eya1/2 genes, which control different aspects of sensory development in invertebrates as well as vertebrates. While So and Eya can induce ectopic eyes in *Drosophila*, the ability of their vertebrate homologues to induce placodes in non-placodal ectoderm has not been explored. Here we show that Six1 and Eya2 are involved in ectodermal patterning and cooperate to induce preplacodal gene expression, while repressing neural plate and neural crest fates. However, they are not sufficient to induce ectopic sensory placodes in future epidermis. Activation of Six1 target genes is required for expression of preplacodal genes, for normal placode morphology and for placode-specific Pax protein expression. These findings suggest that unlike in the fly where the Pax6 homologue Eyeless acts upstream of Six and Eya, the regulatory relationships between these genes are reversed in early vertebrate placode development.

Introduction

In *Drosophila*, *sine oculis* (*so*) and *eyes absent* (*eya*) are nuclear factors that play a key role during compound eye development. Together with seven other transcriptional regulators they form the retinal determination (RD) network, a complex gene regulatory network that controls photoreceptor cell specification in the eye-antennal disc (reviewed in Treisman, 1999; Kumar and Moses, 2001; Donner and Maas, 2004; Pappu and Mardon, 2004). These genes have been placed into a functional network because of their overlapping expression patterns (Bessa et al., 2002) as well as results of loss- and gain-of-function experiments (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994; Serikaku and O'Tousa, 1994; Halder et al., 1995; Bonini et al., 1997; Shen and Mardon, 1997; Czerny et al., 1999; Seimiya and Gehring, 2000; Weasner et al., 2007) and biochemical data (Chen et al., 1997; Pignoni et al., 1997; Czerny et al., 1999; Niimi et al., 1999; Punzo et al., 2002; Ostrin et al., 2006). Like other members of the RD network, So and Eya function is required for normal eye formation and they have the unique ability to induce ectopic eyes when misexpressed in non-retinal tissue and appear to act synergistically (Bonini et al., 1997; Pignoni et al., 1997; Seimiya and Gehring, 2000; Salzer and Kumar, 2009).

In the eye-antennal disc, *so* and *eya* are downstream of Eyeless (Ey) and Twin of Eyeless (Toy) (Halder et al., 1998; Niimi et al., 1999; Bui et al., 2000; Punzo et al., 2002); they act as a complex to activate downstream target genes and positively feed back on *ey* expression itself (Pignoni et al., 1997). As in the fly, vertebrate homologues of the RD network play an important role in eye development but in addition control different aspects of ear, olfactory and sensory ganglia formation including neurogenesis and proliferation (reviewed in Kawakami et al., 2000; Wawersik and Maas, 2000; Hanson, 2001; Donner and Maas, 2004; Silver and Rebay, 2005; Kumar, 2009). They are therefore considered to be key players in controlling cell fate determination in the cranial sensory nervous system, although their regulatory relationship is not always similar to that described in *Drosophila* (for detailed discussion see: Donner and Maas, 2004; Kumar, 2009). In vertebrates, the cranial sensory nervous system largely arises from specialised epithelia, the sensory placodes (reviewed in Baker and Bronner-Fraser, 2001; Streit, 2004; Schlosser, 2006; Streit, 2007). Even before these become morphologically distinct, *so* and *eya* homologues (*Six1* and *-4*; *Eya1* and *-2*) identify most, if not all, placode progenitor cells in what has been called the preplacodal region (PPR) located next to the anterior neural plate (Mishima and Tomarev, 1998; Esteve and Bovolenta, 1999; Sahly et al., 1999; Kobayashi et al., 2000; Pandur and Moody, 2000; Streit, 2002; McLarren et al., 2003; Bessarab et al., 2004; Bhattacharyya et al., 2004; Schlosser and Ahrens, 2004; Litsiou et al., 2005; Xu et al., 2008). Interestingly, their expression begins before that of the *ey* homolog *Pax6* or of any other member of the *Pax* gene family later found in

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different placodes (Pax2: epibranchial and otic; Pax3: trigeminal; Pax6: lens and olfactory; Li et al., 1994; Stark et al., 1997; Groves and Bronner-Fraser, 2000; Bhattacharyya et al., 2004). These observations suggest that Six and Eya proteins may play a crucial role during early sensory progenitor specification and, unlike in *Drosophila*, may act upstream of Pax genes.

Indeed, a recent study in *Xenopus* revealed that Six1 function is required for cells to acquire preplacodal character and that its misexpression leads to upregulation of genes specific for placode precursors at the expense of neural crest and epidermis (Brugmann et al., 2004). Likewise, in Six1 and -4 compound mutant mice the olfactory placode does not form (Chen et al., 2009), consistent with an early, synergistic function of both genes before placode formation. However, it has not yet been tested whether Six and Eya proteins are at the top of the genetic cascade that controls sensory fates by regulating the onset of placode-specific Pax gene expression or whether they can induce ectopic placodes, as may be expected from their eye-inducing ability in the fly. The Six family member *Six3* is expressed early in the lens territory, regulates *Pax6* (Liu et al., 2006) and has been shown to induce ectopic lens-like structures in fish (Oliver et al., 1996). However, this lens-inducing ability is confined to the PPR suggesting that this territory possesses special properties distinct from the remaining non-neural ectoderm.

In support of this idea, recent evidence shows that the acquisition of preplacodal character is an essential step for placode induction: only preplacodal cells are competent to form placodes in response to the appropriate inducing signals (Martin and Groves, 2006). Furthermore, all cells within the placode territory have a common developmental potential: irrespective of their later fate they are initially specified as lens and lens formation must be suppressed for other neurogenic placodes to develop (Bailey et al., 2006). Together, these observations suggest that the PPR has unique properties and that Six and Eya proteins may play an important role for the acquisition of PPR character.

Here we address the question of whether Six1 and Eya2 are sufficient to confer PPR properties to non-placodal cells, whether they are sufficient to induce ectopic placodes and whether they act upstream of placode-specific Pax gene expression. We show that in chick, as in the fly, Six1 and Eya2 act synergistically: together they promote preplacodal gene expression while suppressing neural and neural crest cell fates. While activation of Six1 target genes is required for the specification of placode progenitors, Six1 and Eya2 are not sufficient to impart preplacodal properties (placode competence or lens specification) to cells that normally do not contribute to the sensory nervous system. Likewise, combined expression of Six1 and Eya2 does not induce ectopic placodes. However, unlike in the fly, Six1 appears to act upstream of placode-specific *Pax* gene expression: activation of Six1 target genes is required for their expression and for ectodermal cells to acquire placode morphology.

Materials and methods

Expression constructs and morpholinos

The coding region of human Six1 (Boucher et al., 1996) and chick Eya2 (Mishima and Tomarev, 1998) were cloned into the pCAB-IRES-GFP (Niwa et al., 1991). Engrailed-Six1HD in pCS2 was a kind gift from Dr Sally Moody (Brugmann et al., 2004). Fluorescein-coupled morpholinos leading to deletion of exon3 or exon6 of chick Eya2 and respective control morpholinos were described previously (Mende et al., 2008).

Embryo culture, electroporation and explant cultures

Fertile hens' eggs (Henry Stewart) were incubated at 38 °C to reach stage HH3$^+$/4 or 5–6 (Hamburger and Hamilton, 1951) and cultured according to a modified version of New's method (New, 1955; Stern and Ireland, 1981). Electroporation of expression constructs at HH3$^+$/4 and morpholinos at HH5–6 was conducted as previously described (McLarren et al., 2003; Mende et al., 2008). After overnight culture, embryos were fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 15 min for immunohistochemistry or overnight at 4 °C for in situ hybridisation.

To assay the ability of Six1 and Eya2 to confer lens specification and FGF2 responsiveness to non-placodal cells, electroporated area pellucida or area opaca epiblast visualised by GFP fluorescence was dissected after 12 h using tungsten needles. The placode territory without underlying mesoderm and endoderm was dissected from stage HH6 embryos as positive control. Explants were kept in Tyrode's saline on ice, until collagen cultures were set up as previously described (Bailey et al., 2006). Explants were grown in vitro at 37 °C in the presence of FGF2 (250 ng/ml; R&D systems; Martin and Groves, 2006) for 20 h to assay otic marker expression or absence of any growth factors for 60–72 h to assay lens specification. Explants were then fixed for 15 min in 4% PFA in PBS for immunohistochemistry.

Whole-mount in situ hybridisation and immunocytochemistry

Whole-mount in situ hybridisation was performed using digoxigenin (DIG)-labelled antisense RNA probes as previously described (Streit et al., 1998). The following plasmids were used to generate DIG-labelled antisense riboprobes: Dlx5 (McLarren et al., 2003), Eya2 (Mishima and Tomarev, 1998), Gata3 (Sheng and Stern, 1999), GnRH1 (a gift from Dr Ian Dunn), Pax2 (a gift from Dr Martyn Goulding), Pax6 (Li et al., 1994), Pax7 (Basch et al., 2006), Raldh3 (Blentic et al., 2003), Six1 (a gift from Dr Guillermo Oliver), Six4 (Esteve and Bovolenta, 1999) and Sox2 and Sox3 (Uwanogho et al., 1995).

Immunocytochemistry for GFP was performed using a polyclonal anti-GFP antibody (Molecular Probes; 1:2000 in PBS containing 5% sheep serum, 1% Triton X-100) followed by an HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; 1:500 in PBS containing 5% sheep serum, 1% Triton X-100). Samples were then processed for cryosectioning.

Immunocytochemistry on cryosections was performed using polyclonal antibodies against chick δ-crystallin (a gift from Dr J. Piatigorsky), GFP (Molecular probes) and chick Pax2 (Zymed), and monoclonal antibodies against phospho-Histone H3 (Imgenex), Pax3 and Pax6 (Developmental Hybridoma Bank). The appropriate Alexafluor 488- and 594-coupled secondary antibodies were purchased from Molecular Probes; nuclei were stained by DAPI (Molecular Probes).

To quantify the number of morpholino or GFP carrying cells expressing Pax2, Pax6 and Pax3 cells in each embryo, digital images from each section were taken after immunostaining using a Leica TCS SP5 confocal microscope. For each section, the total number of morpholino or GFP carrying cells was determined by counting the green fluorescent cells with visible nuclei (MO or GFP/DAPI$^+$). The number of Pax2$^+$, Pax3$^+$ and Pax6$^+$ cells among the morpholino carrying cells was determined by counting the MO or GFP/Pax2, -3 or -6/DAPI+ cells. An unpaired *t*-test was performed to determine the statistical significance between splice-blocking morpholinos and control morpholinos and between engrailed-Six1HD and GFP electroporated cells.

Results

Six1 and Eya2 promote preplacodal gene expression but are not sufficient to induce ectopic placodes

To assess whether Six and Eya proteins alone or in combination are sufficient to induce ectopic sensory placodes, as may be expected from their eye-inducing ability in *Drosophila*, or to impart preplacodal

character we used gain-of-function experiments in the chick embryo. After electroporation of primitive streak stage (HH3$^+$/4) embryos with full length Six1 or Eya2, no changes were observed in markers specific for the neural plate (*Sox2*: Six1 $n=7$, Eya2 $n=6$), neural crest cells (*Pax7*: Six1 $n=7$, Eya2: $n=5$), non-neural ectoderm (*Dlx5*: Six1 $n=11$, Eya2: $n=7$; Gata3: Six1 $n=7$) or the placode territory itself (*Six4*: Six1 $n=11$, Eya2: $n=8$; *Eya2*: Six1 $n=10$) (data not shown). However, when misexpressed together, Six1 and Eya2, but not GFP (controls *Sox2* $n=8$; *Sox3* $n=7$; *Pax7* $n=7$; *Six4* $n=11$; *Dlx5* $n=11$; *Gata3* $n=5$; Figs. 1A–C, A′–C′, a–c, G–I, G′–I′, g–i), repress *Sox2* and *Sox3* (13/16, 8/11; Figs. 1D, D′, d, E, E′, e) and *Pax7* (16/27; Figs. 1F, F′, f), while inducing ectopic expression of the PPR marker *Six4* in the future epidermis and in the extraembryonic region (13/20; Figs. 1J, J′, j). In addition, misexpression of Six1 and Eya2 leads to non-cell autonomous upregulation of *Dlx5* (7/9; Figs. 1K, K′, k, arrow heads) and *Gata3* (6/10; Figs. 1L, L′, l, arrow head).

To determine whether Six1 and Eya2 are sufficient to induce ectopic placodes, as might be expected from their eye-inducing ability in the fly, we tested for ectopic expression of placode-specific markers after Six1 and Eya2 misexpression. We never observed ectopic expression of *Pax2* (otic and epibranchial; $n=9$; Figs. 2A, A′), *Pax6* (lens; $n=14$; Figs. 2B, B′) or *GnRH1* and *Raldh3* (olfactory; $n=5$ and $n=6$; Figs. 2C, C′, D, D′).

It has previously been suggested that high levels of Six1 and Eya1 expression promote proliferation, while low levels promote differentiation into otic neurons (Schlosser et al., 2008). To test whether

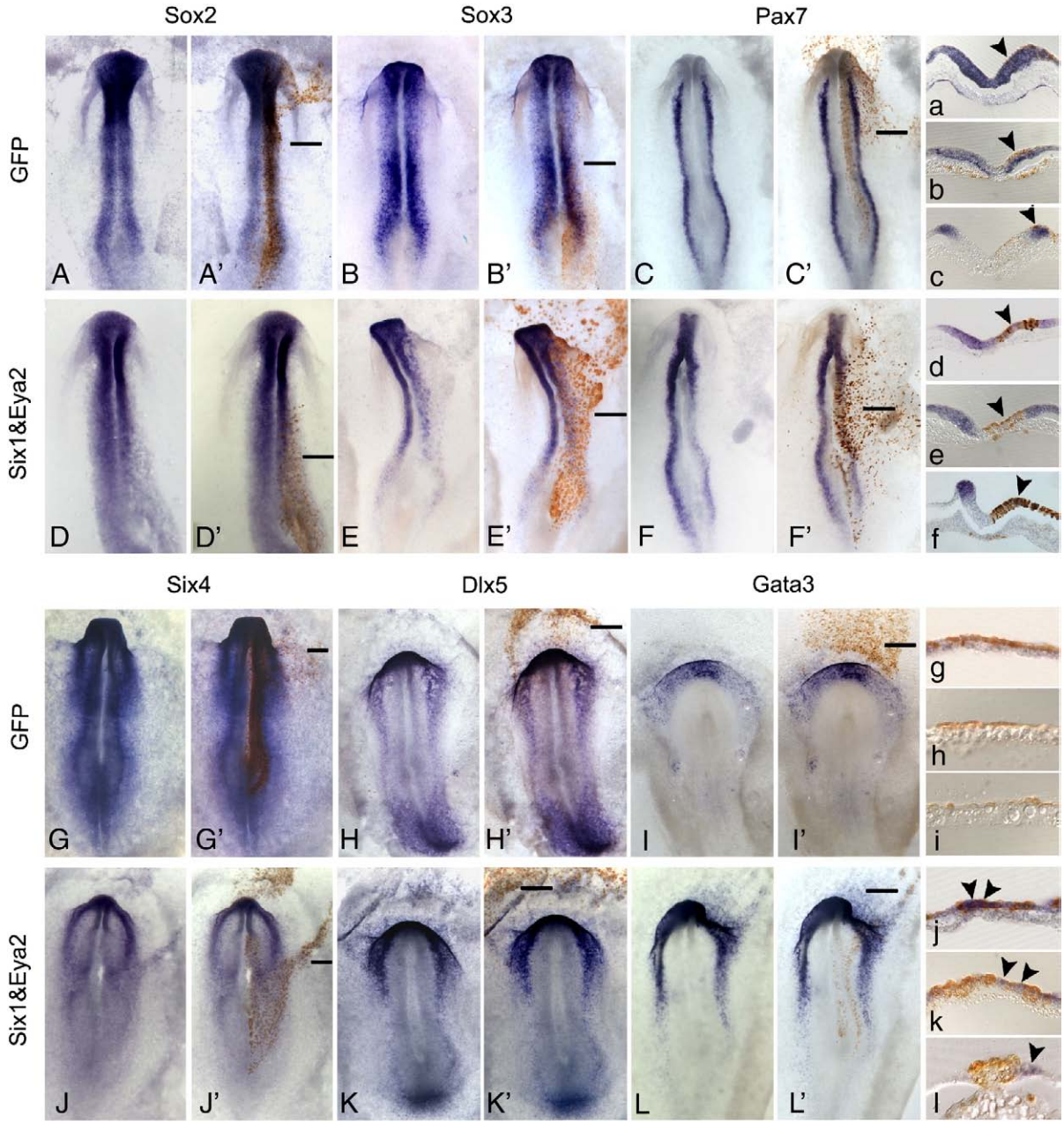

Fig. 1. Six1 and Eya2 promote preplacodal properties. GFP (A–C, A′–C′, G–I, G′–I′, a–c, g–i) or Six1 and Eya2 (D–F, D′–F′, d–f, J–L, J′–L′, j–l) were electroporated into primitive streak stage embryos. After overnight culture, the expression of neural, neural crest, preplacodal and non-neural ectoderm markers was assessed by in situ hybridisaton (blue) followed by GFP immunohistochemistry (brown) to visualise transgene carrying cells. Panels A–L show embryos before GFP staining and panels A′–L′ the same embryos thereafter. Six1/Eya2 misexpression suppresses *Sox2* (D, D′, d), *Sox3* (E, E′, e) and *Pax7* (F, F′, f) while upregulating *Six4* (J, J′, j), *Dlx5* (K, K′, k) and *Gata3* (L, L′, l). GFP misexpression does not affect the expression of any marker (A–C, A′–C′, G–I, G′–I′, a–c, g–i). Arrow heads in a–f and j point to electroporated cells (brown), arrow heads in k and l to cells that non-autonomously upregulate *Dlx5* and *Gata3* expression (blue), respectively. Lines in A′–L′ correspond to the level of the sections in a′–l′.

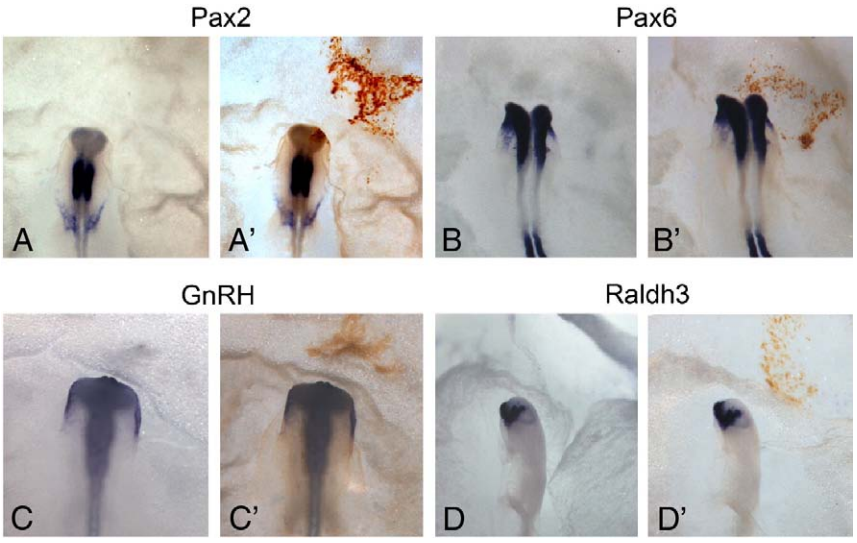

Fig. 2. Six1 and Eya2 do not promote ectopic placode formation. Six1 and Eya2 were electroporated into primitive streak stage embryos. After 20–24 h, the expression of otic (A, A′), lens (B, B′) and olfactory markers (C, C′, D, D′) was assessed by in situ hybridisation (blue; A–D). Electroporated cells were visualised by GFP immunohistochemistry (brown; A′–D′). Upregulation of *Pax2* (A, A′), *Pax6* (B, B′), *GnRH* (C, C′) and *Raldh3* (D, D′) was never observed.

Six1 and Eya2 increase proliferation in epiblast cells we compared phospho-Histone H3 staining in GFP (control; $n=7$) and Six1/Eya2 ($n=7$) electroporated embryos. No difference was observed (data not shown) suggesting that the failure to differentiate into placodes is not due to increased proliferation of cells carrying exogenous Six1 and Eya2.

These results suggest that while Six1 and Eya2 act together to promote preplacodal character, they cannot induce placode-specific gene expression in non-placodal ectoderm.

Six1 and Eya2 do not account for all PPR properties

The PPR is a unique part of the neurula stage ectoderm: it is the only region responsive to otic inducing signals and it is initially specified as lens (Bailey et al., 2006; Martin and Groves, 2006). We therefore assessed whether Six1 and Eya2 are sufficient to confer these properties to cells not fated to become placodes. Six1 and Eya2 were misexpressed at the border of the embryonic and extraembryonic region at gastrula stages ($HH3^+/4$). After overnight culture, the electroporated tissue was excised and cultured in the presence or absence of the otic inducing signal FGF2 for 20–24 h (Fig. 3A). PPR from HH6 stage embryos was used as a positive control. While 87.5% of PPR explants expressed the otic marker Pax2 (7/8; Figs. 3B–D), neither FGF2 treated ($n=14$; Figs. 3E–G) nor untreated ($n=11$; data not shown) tissue carrying exogenous Six1 and Eya2 did. Likewise, unlike endogenous PPR from HH6 (8/9; Figs. 3H–J), Six1 and Eya2 electroporated epiblast cultured for 60–72 h in the absence of growth factors does not acquire lens character as assessed by δ-crystallin expression ($n=15$; Figs. 3K–M). These results show that although Six1 and Eya2 can induce PPR markers in non-placodal ectoderm (see above), together they are not sufficient to impart PPR properties to cells not normally fated to become placodes.

Loss of Eya2 causes a moderate decrease of Pax2 expression

While *Six1* and *Six4* are initially weakly expressed in the neural plate before becoming confined to the PPR, *Eya2* is confined to the PPR from the onset of its expression. Although weak expression of Eya1 has been reported in the anterior PPR (Ishihara et al., 2008), in our hands this transcript cannot be detected. We therefore asked whether Eya2 is required for PPR specification and placode formation. Two different morpholinos were electroporated into placode precursors at primitive streak to head process stages ($HH3^+$–5) and the expression of PPR and placode markers was assessed after 15–24 h culture. No change in *Six1* or *Six4* expression was observed (*Six1* $n=10$; *Six4* $n=8$; data not shown), and Eya2 MO ($n=13$) carrying cells appear to integrate into the otic placode to the same extent as control MO electroporated cells ($n=9$ for each). However, a very moderate reduction of Pax2 protein in the otic placode is observed in Eya2 MO carrying cells when compared to controls (control: $99.66\% \pm 0.34$, Eya2 MO $89.33\% \pm 1.19$ of all electroporated cells are $Pax2^+$, $p=0.021$; Supplementary Fig. 1). When Eya2 MOs are targeted to the lens territory (Eya2 MO $n=9$; control MO $n=10$) no changes in the early lens marker *Pax6* are observed.

We next investigated whether Eya2 is required for PPR properties, such as competence to respond to otic inducing signals or lens specification. Eya2 or control MOs were electroporated into placode precursors, the targeted tissue was excised and cultured overnight in the presence or absence of FGF2 and assayed for the expression of the otic marker Pax2 after FGF2 treatment and for the early lens marker Pax6. There is no difference in Pax2 induction by FGF2 or in the number of $Pax6^+$ (i.e., lens specified) cells between Eya2 and control MO electroporated explants.

In summary, while Eya2 knock-down leads to a moderate reduction in expression of the otic marker Pax2, PPR character remains unchanged when Eya2 is reduced.

Six1 regulates gene expression at the neural plate border

Unlike Eya proteins, Six1 directly binds to specific DNA target sequences via its homeodomain and acts as an activator in association with Eya proteins (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003; Hu et al., 2008). Our results above show that misexpression of Six1 and Eya2 leads to upregulation of PPR specific gene expression (Fig. 1). To investigate whether activation of Six1 target genes is required, we used an expression construct where the homeodomain of Six1 is fused to a constitutive repressor (EnR). Electroporation of EnR-Six1, but not of GFP (Figs. 4E–E′, F–F′) at gastrula stages ($HH3^+/4$) leads to loss of the preplacodal markers *Six4* (8/10; Figs. 4A–A′, a) and *Eya2* (4/8;Figs. 4B–B′, b), while *Dlx5* expression in the placode territory remains unchanged ($n=6$; data not shown). Occasionally, we observe an expansion of the neural marker *Sox2* into the non-neural ectoderm (6/11;Figs. 4C–C′, c) suggesting that when Six1 target genes are repressed, cells at the neural plate border can acquire

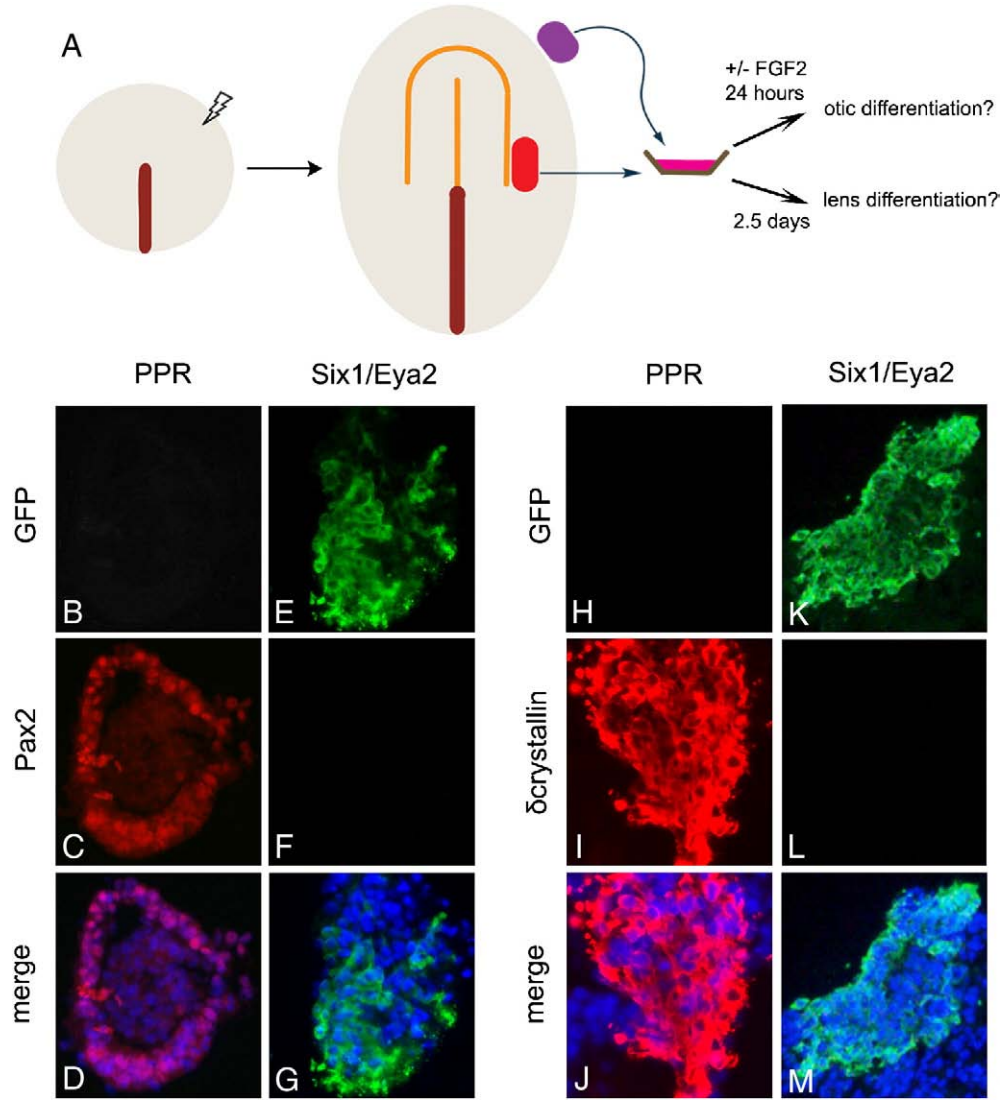

Fig. 3. Six1 and Eya are not sufficient to confer preplacodal properties to non-placodal cells. (A) To assess whether Six1 and Eya2 impart responsiveness to otic inducing signals or lens specification to cells that normally do not form sensory placodes, Six1 and Eya2 were electroporated into the ectoderm at HH3$^+$/4; embryos were grown until they had reached HH5/6 and the electroporated cells were isolated and cultured in the presence of FGF2 for 24 h to assess otic markers or in the absence of growth factors for 2.5 days to assess lens markers. PPR from HH5/6 embryos was used as positive control. In the presence of FGF2, PPR explants (B–D) express the otic marker Pax2 (red in C, magenta in D), while Six1/Eya2 electroporated ectoderm does not (E–G; green). After 2.5 days in culture (H–J), PPR explants express lens specific δ-crystallin (red in I, J). However, Six1/Eya2 electroporated ectoderm (K–M, green) is negative for δ-crystallin. D, G, J and M show nuclei stained with DAPI.

neural character. In contrast, repression of Six1 target genes in the anterior neural folds, which normally do not generate neural crest cells, leads to expansion of the neural crest marker *Pax7* (7/11;Figs. 4D–D′, d) and we occasionally observe lateral expansion of *Pax7* into the placode territory.

Thus, repression of Six1 target genes and misexpression of Six1/Eya2 show opposite phenotypes (compare Figs. 1 and 4), suggesting that in the context of sensory precursor specification Six1 acts as an activator. Preplacodal expression of *Six4* and *Eya2* is dependent on Six1 or its targets, while preventing the formation of neural and neural crest cells in tissue that normally forms placodes may depend on activation of a repressor.

Six1 regulates Pax gene expression in placode progenitors and placodal morphology

In placode precursors, Six and Eya genes are expressed prior to the onset of placode-specific Pax gene expression and prior to the formation of morphological placodes. Our results above show that misexpression of Six1/Eya2 is not sufficient to induce placodes in non-placodal ectoderm; however, activation of Six1 targets may be required. Indeed, misexpression of EnR-Six1 leads to severe malformation of the otic placode ($n=13$; Figs. 5A b, b′, b″, c c′), which becomes split into multiple 'cuplets'. Cells carrying the transgene fail to acquire columnar morphology typical of the placode and do not express Pax2 (70.2%; $n=7$; Figs. 5A b, b′, b″, d). EnR-Six1 expressing cells appear to cluster and disrupt the organisation of the otic placode. Likewise, when misexpressed in the trigeminal and lens territory, EnR-Six1 cells fail to express Pax3 (44.15%; $n=7$;Figs. 5B, b, b′, b″, c) and Pax6 (49.55%; $n=7$;Figs. 5C, b, b′, b″, c), respectively. In contrast, control electroporated embryos never show any placode defects or significant loss of Pax protein expression (Pax2: 9.96%, Figs. 5A a, a′, a″, d $n=6$; Pax3: 17.4%, Figs. 5B a, a′, a″, c, $n=4$; Pax6: 5.4%, $n=7$, Figs. 5C a, a′, a″, c). Statistical analysis shows that the differences observed between EnR-Six1 and GFP misexpression are significant (Pax2: $p=0.0000016$, Fig. 5A d; Pax3: $p=0.0003$, Fig. 5B c; Pax6: $p=0.0000027$, Fig. 5C c). Thus, activation of Six1 target genes is required for the expression of placode-specific Pax proteins. However, whether Six proteins directly activate Pax gene transcription or whether intermediate factors are required remains to be

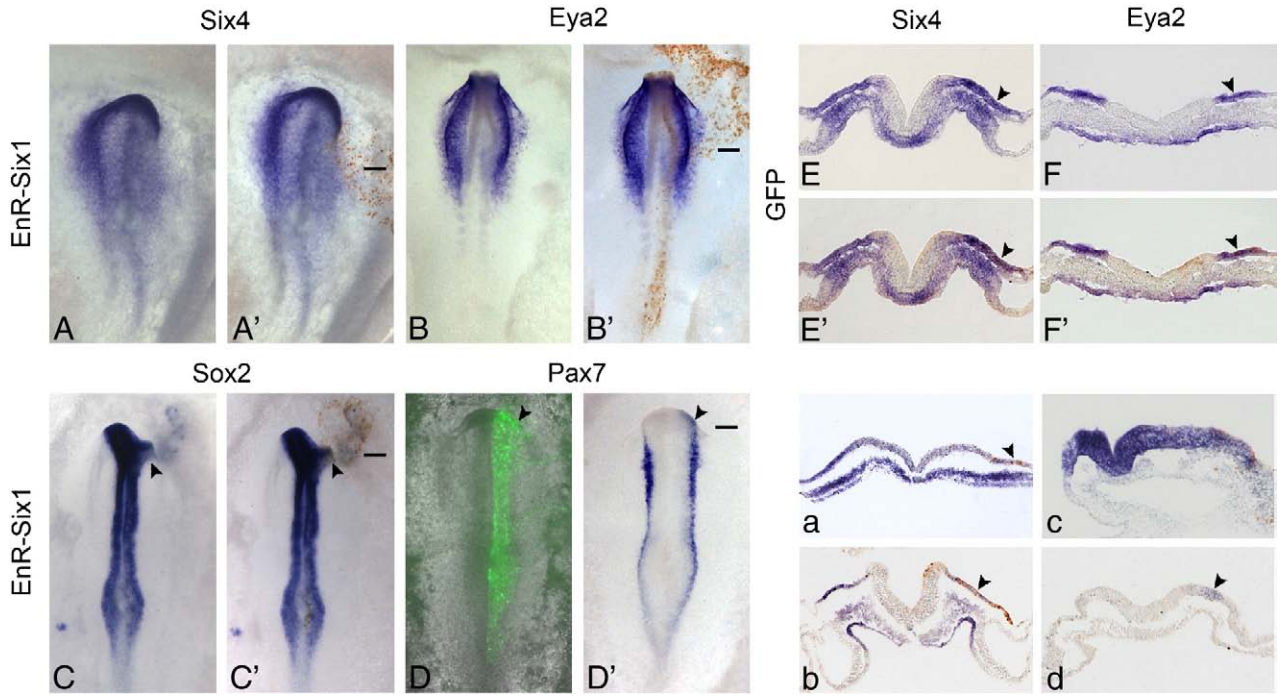

Fig. 4. Activation of Six1 target genes is required for preplacodal gene expression. EnR-Six1 (A–D, A′–D′) or GFP (E, E′, F, F′) was misexpressed at primitive streak stages. The expression of preplacodal (A, A′, a, B, B′, b, E, E′, F, F′), neural (C, C′, c) and neural crest (D, D′, d) was assessed at HH7–8 by in situ hybridisation (blue; A–C, D′, E, F), followed by GFP immunohistochemistry (brown; A′–C′, E′, F′) to visualise electroporated cells. Panels A–C, E, F show embryos before GFP staining, panels A′–C′, E′, F′ thereafter. (D) Fluorescent image to visualise EnR-Six1 carrying cells; D′ shows the same embryo as in D. Repression of Six1 target genes results in a loss of *Six4* (A, A′, a) and *Eya2* (B, B′, b) expression, while *Sox2* (C, C′, c; arrow head) is expanded laterally and *Pax7* expands into the anterior neural folds (D, D′, d; arrow head). Misexpression of GFP does not change *Six4* (E, E′) or *Eya2* (F, F′) expression. Lines in A′–D′ correspond to the level of the sections shown in a–d.

elucidated. Finally, our results also show that activation of Six1 targets is required for cells to integrate into the placode and to acquire placode morphology.

Discussion

Six and Eya genes have been implicated in multiple aspects of sensory organ development from vertebrates to flies (reviewed in Kawakami et al., 2000; Wawersik and Maas, 2000; Hanson, 2001; Donner and Maas, 2004; Pappu and Mardon, 2004; Silver and Rebay, 2005; Kumar, 2009). They control proliferation and survival of neuronal and sensory progenitors as well as the expression of neuronal determination and differentiation genes in different sensory placodes (Xu et al., 1999; Li et al., 2003; Zheng et al., 2003; Ozaki et al., 2004; Zou et al., 2004; Bricaud and Collazo, 2006; Zou et al., 2006; Schlosser et al., 2008; Chen et al., 2009). However, *Eya1/2* and *Six1/4* are already expressed in placode progenitors long before morphological placodes are visible or neurogenesis takes place. Recent findings in *Xenopus* suggest that at this stage they are part of a complex regulatory network of transcription factors that patterns the embryonic ectoderm to subdivide it into neural, neural crest, placode precursors and epidermis (Brugmann et al., 2004). Our findings are in agreement with these observations, confirming that Six1 and its co-activator Eya2 promote placode precursor identity, while repressing neural and neural crest fate.

Transcription factors in the placode territory

Six1 is a DNA binding factor that acts as a transcriptional activator when interacting with members of the Eya family, but as a repressor in the presence of Groucho proteins (Hu et al., 2008, Brugmann et al., 2004). Here, we show that ectopic expression of Six1 and Eya2 promotes placode progenitor fate, while simultaneously repressing neural and neural crest cells. Conversely, a constitutive repressor form of Six1 leads to the loss of preplacodal gene expression (*Six4*, *Eya2*), while neural and neural crest markers are expanded. In particular, we find that when Six1 target genes are repressed *Pax7* extends into the anterior neural folds, which normally never generate neural crest cells. These results suggest that some Six1/Eya2 targets act to repress neural and neural crest specific genes, while preplacodal genes depend on Six1 activator function. In *Xenopus*, the neural crest marker *FoxD3* is also repressed in the presence of exogenous Six1; however, this appears to be mediated through its association with Groucho (Brugmann et al., 2004). Thus, different neural crest specifiers are differentially regulated by Six1 and it remains to be elucidated whether any of these regulatory relationships are direct or indirect.

We did not observe similar effects on ectodermal patterning using an Eya2 knock-down approach. The most likely explanation for this difference is that preplacodal expression of *Six4* compensates for the loss of the Six1/Eya2 complex. Unlike Six1, Six4 contains a transactivation domain and may thus activate targets independent of Eya2. Since the consensus binding sites for Six1 and -4 are virtually identical (Kawakami et al., 1996; Spitz et al., 1998; Suzuki-Yagawa et al., 1992), EnR-Six1 is likely to repress target genes of both factors and prevent compensation by Six4.

Dlx genes are present in the neural plate border prior to the onset of preplacodal genes (Akimenko et al., 1994; Beanan and Sargent, 2000; Feledy et al., 1999; Kaji and Artinger, 2004; Luo et al., 2001; Pera et al., 1999). They are required for *Six1* and *Eya1* expression (Solomon and Fritz, 2002; Woda et al., 2003) and sufficient to induce *Six4* transcripts (McLarren et al., 2003). Misexpression of preplacodal genes leads to non-cell autonomous, ectopic *Dlx5* activation in the future epidermis (Brugmann et al., 2004) (this study), while it is repressed in cells carrying exogenous Six/Eya. This finding suggests that Six1/Eya2 activate a Dlx5 repressor as well as a signal that non-cell autonomously activates *Dlx5* in neighbouring cells. Thus, while Dlx genes initially promote Six and Eya gene expression, a negative feedback loop seems to repress Dlx genes in forming placodes.

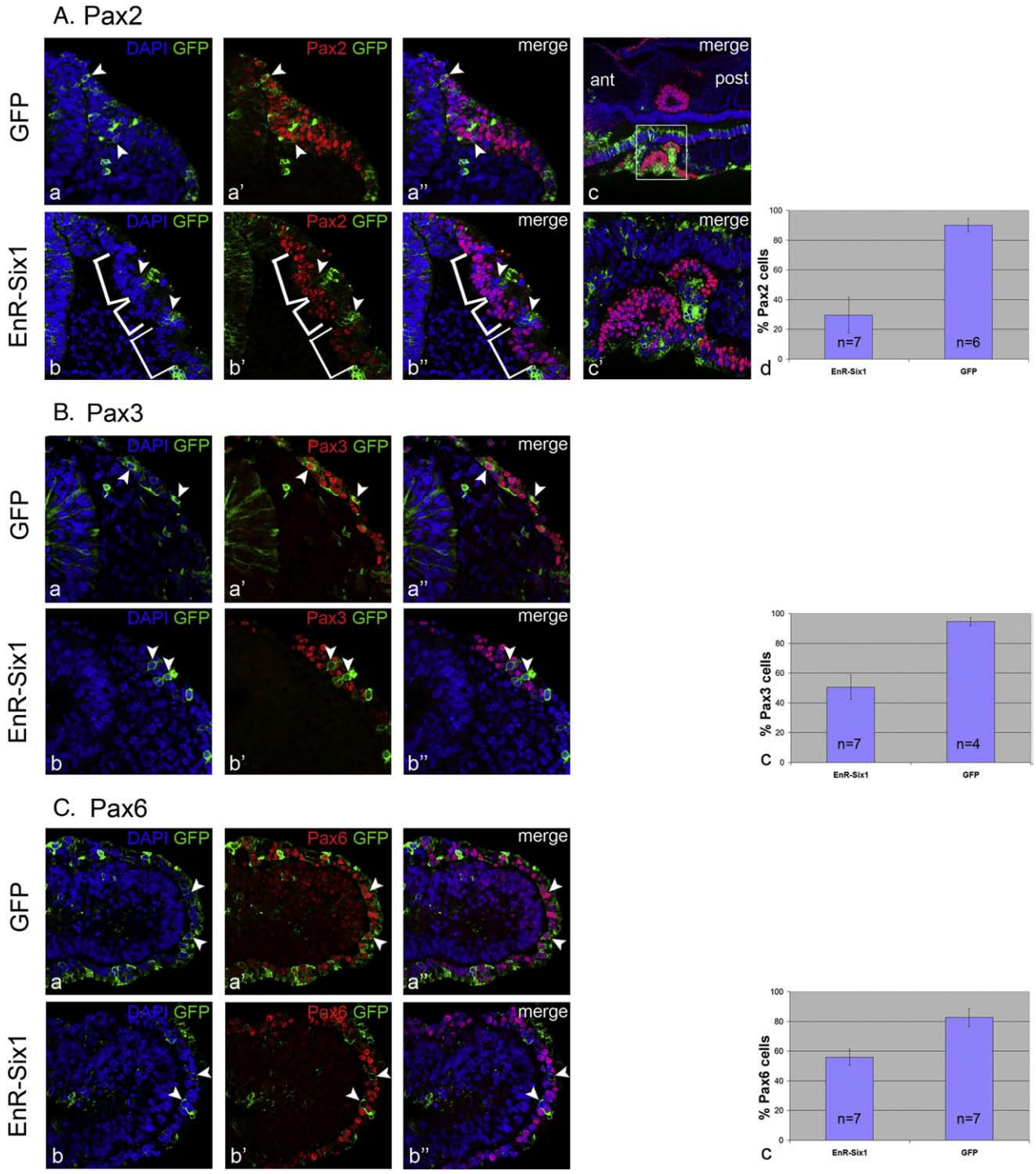

Fig. 5. Activation of Six1 target genes is required for placode-specific Pax protein expression and placode formation. EnR-Six1 or GFP was electroporated into the future otic (A), trigeminal (B) or lens (C) region; at HH9^{+}–11 the expression of Pax2 (A), Pax3 (B) and Pax6 (C) was assessed by immunocytochemistry in transverse sections. (A) In the otic placode, cells misexpressing GFP (white arrow heads in a–a″, green) continue to be Pax2^{+} (red in a′, magenta in a″). However, in the presence of EnR-Six1 (white arrow heads in b–b″, green), Pax2 expression is lost (red in b′, magenta in b″) and otic placode morphology is disrupted. White brackets indicate the formation of multiple small otic cups. (C, c′) Dorsal view (anterior towards the left) of an embryo electroporated with EnR-Six1 into the left otic region (green) and stained for Pax2 (magenta). C′ shows a higher magnification of the area indicated by the white square in c. Note the disruption of otic morphology and formation of multiple smaller cups. To quantify the effect, the percentage of Pax2^{+} cells among electroporated cells was counted in 7 EnR-Six1 and 6 GFP expressing embryos (d). Pax2 expression is reduced to approximately 30% when Six1 target gene activation is inhibited ($p = 1.6 \times 10^{-6}$). (B) In the trigeminal placode, cells misexpressing GFP (white arrow heads in a–a″, green) continue to be Pax3^{+} (red in a′, magenta in a″). However, in the presence of EnR-Six1 (white arrow heads in b–b″, green), Pax3 expression is lost (red in b′, magenta in b″). The effect was quantified by counting Pax3^{+} cells among electroporated cells in the trigeminal region in 7 EnR-Six1 and 4 GFP expressing embryos (c). Pax3 expression is reduced to approximately 50% when Six1 target gene activation is inhibited ($p = 3.0 \times 10^{-4}$). (C) In the lens placode, cells misexpressing GFP (white arrow heads in a–a″, green) continue to be Pax6^{+} (red in a′, magenta in a″). However, in the presence of EnR-Six1 (white arrow heads in b–b″, green), Pax6 expression is lost (red in b′, magenta in b″). The effect was quantified by counting Pax6^{+} cells among electroporated cells in the lens placode in 7 EnR-Six1 and 7 GFP expressing embryos (c). Pax6 expression is reduced to approximately 50% when Six1 target gene activation is inhibited ($p = 2.7 \times 10^{-6}$).

One surprising difference between previous *Xenopus* and chick results is that Six1 can act alone to pattern the ectoderm in *Xenopus*, while in chick misexpression of Six1 together with the co-activator Eya2 is required. The latter is reminiscent to the situation in *Drosophila*, where the eye-inducing activity of So and Eya alone is relatively low, but increases considerably when both are co-expressed in non-retinal tissue (Bonini et al., 1997; Pignoni et al., 1997; Seimiya and Gehring, 2000; Salzer and Kumar, 2009). The main differences between the *Xenopus* and chick experiments are timing and the tissue expressing exogenous constructs: while in *Xenopus* Six1 was injected into ventro-lateral blastomeres at the 32-cell stage, misexpression experiments in chick were performed at late gastrula stages, shortly before *Six1* and *Eya2* become normally expressed. Frog ventro-lateral blastomeres not only contribute to neural crest and placode cells, but also to mesodermal and endodermal derivatives, which come to underlie the placode territory (Moody, 1987). It has previously been shown that mesodermal signals are required for the induction of the placode territory (Litsiou et al., 2005). It is therefore possible that changes in the mesoderm, where the Six/Eya network participates in somite formation (Heanue et al., 1999), indirectly influence the overlying ectoderm. Another possibility is that *Eya1*, which is already expressed at early gastrula stages in *Xenopus* (David et al., 2001), may interact with exogenous Six1 to affect patterning of the ectoderm, whereas *Eya1* and -2 are absent at this stage in the chick (McLarren et al., 2003; Litsiou et al., 2005; Ishihara et al., 2008).

Are Six1 and Eya2 specifiers for placodal progenitors?

Thus, Six and Eya proteins appear to be part of a regulatory network of transcription factors that controls cell fate specification at the border of the neural plate and ensures the segregation of neural, neural crest, placodal and epidermal fates. Six1 and Eya2 promote preplacodal gene expression, but are they sufficient to endow placode progenitor properties onto cells that normally never contribute to placodes? Recent evidence suggests that to generate sensory placodes, ectodermal cells first have to acquire a 'preplacodal state', which allows them to respond to placode-specific inducing signals (Martin and Groves, 2006). In addition, preplacodal cells initially share a common developmental potential and are initially specified as lens, before lens suppressing and neurogenic placode inducing signals generate placode diversity (Bailey et al., 2006). Here we show that while misexpression of Six1 and its cofactor Eya2 can induce preplacodal gene expression, their expression alone does not account for all properties of placode progenitors. Cells expressing exogenous Six1/Eya2 cannot respond to FGF2 to activate otic markers, nor are they lens specified.

Based on the eye-inducing activity of So and Eya in *Drosophila*, we predicted that Six1 and Eya2 may induce ectopic placodes in tissue not normally fated to contribute to the cranial sensory nervous system. However, we find that misexpression of both genes does not lead to ectopic morphological placodes and does not initiate the expression of placode-specific gene expression.

Together, these results suggest that additional factors are required for cells to acquire preplacodal character and for the induction of ectopic placodes in future epidermis. Indeed, in *Drosophila* Six1 binding partners in addition to Eya and Groucho proteins have been identified (Giot et al., 2003); however, their role in vertebrate placode formation has not been investigated. Finally, we cannot exclude the possibility that more subtle regulation of *Six1* and *Eya2* expression levels is required for ectodermal cells to undergo placode differentiation.

Six1 acts upstream of placode-specific Pax genes

In *Drosophila* the expression and function of *so* and *eya* in the eye are dependent on the function of the Pax6 homologues Ey and Toy. Surprisingly however, a similar regulatory relationship is not conserved in the vertebrate sensory system. While Pax6 has retained its activity as a key regulator of vertebrate eye development, its early expression in the future lens ectoderm depends on direct activation by Six3 via the ectodermal enhancer (Liu et al., 2006). Likewise, in the murine olfactory placode, *Pax6* expression requires Six1 and Six4 function (Chen et al., 2009) and the onset of *Six3* and *Eya1* expression is independent of Pax6 (Purcell et al., 2005). In the otic vesicle, *Pax2*, *Eya1* and *Six1* are expressed in partially overlapping domains, but *Six1* and *Eya1* expression is independent of Pax2, while Eya1 function is required for *Six1* expression (Zheng et al., 2003; Burton et al., 2004). In the preplacodal region, *Six1*, *Six4* and *Eya2* are clearly present prior to the onset of placode-specific expression of *Pax6*, *Pax3* and *Pax2*. Here we show that although ectopic expression of Six1 and Eya2 does not result in the induction of Pax genes, the activation of Six1 downstream targets is required for their expression in the lens, trigeminal and otic placode. Likewise, in zebrafish Six1 morphants loose otic specific expression of *Pax2a* and *Pax2b* (Bricaud and Collazo, 2006). The regulatory elements that control the onset Pax gene expression in sensory placodes have not been identified and therefore it remains to be determined whether their activation by Six genes is direct or intermediate factors are required. While Pax genes are clearly required for sensory organ development in vertebrates, with Pax6 being essential for lens and olfactory placode formation (Fujiwara et al., 1994; Collinson et al., 2001; Collinson et al., 2003), Pax3 for trigeminal (Dude et al., 2009) and Pax2 for otic development (Torres et al., 1996; Burton et al., 2004), these findings suggest that unlike in the fly eye, Six and Eya, but not Pax proteins, are at the top of the genetic hierarchy that specifies sensory precursor cells in vertebrates.

Together our findings propose that while some of the molecular interactions of the RD network are conserved between flies and vertebrates, some of the regulatory relationships have diverged during evolution of the sensory nervous system (for further discussion, see Donner and Maas, 2004; Kumar, 2009). Future studies of sensory placode-specific enhancers will need to clarify the molecular interactions of RD network factors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.09.025.

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